

Amyloid properties of the leader peptide of variant B cystatin C: implications for Alzheimer and Macular Degeneration

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ABSTRACT

Variant B (VB) of cystatin C has a mutation in its signal peptide (A25T), which interferes with its processing leading to reduced secretion and partial retention in the vicinity of the mitochondria. There are genetic evidences of the association of VB with Alzheimer' disease (AD) and age-related macular degeneration (AMD). Here we investigated aggregation and amyloid propensities of unprocessed VB combining computational and *in vitro* studies. Aggregation predictors revealed the presence of four aggregation-prone regions, with a strong one at the level of the signal peptide, which indeed formed toxic aggregates and mature amyloid fibrils in solution. In the light of these results, we propose for the first time the role of the signal peptide in pathogenesis AD and AMD.

HIGHLIGHT BULLETS

Variant B precursor of the cysteine proteinase inhibitor cystatin C has been previously associated with increased risk of developing Alzheimer's Disease and exudative Age-related Macular Degeneration.

We show here that the leader sequence of the variant B precursor cystatin C presents high aggregation and amyloidogenic propensity.

Retention of the leader sequence in the incompletely processed variant can contribute to deleterious protein aggregation linked to pathogenesis of AD and AMD.

1. Introduction

Human Cystatin C belongs to the cystatin superfamily of reversible inhibitors of cysteine proteases encompassing the papain and legumain families [1]. The mature cystatin C is a monomeric protein composed of a polypeptide chain of 120 amino acids (13,343 Da) [2,3]. The general fold of monomeric inhibitors of the cystatin superfamily has been defined by the crystal structure of chicken cystatin. It is composed of a long helix running across a five-stranded antiparallel β -sheet [3,4] (**Fig. 1A**). Cystatin C is produced and found in most tissues and body fluids with particular higher concentrations in the cerebrospinal fluid, owing to expression by the choroid plexus. Cystatin C is involved in many biological functions, ranging from bone reabsorption, modulation of inflammatory response, anti-viral and anti-bacterial properties, cell proliferation and growth, tumor metastasis, and to astrocyte differentiation during mouse brain development [5–9].

Wild type (WT) cystatin C has been found in amyloid deposits in the brain arteries of elderly people [10,11], while the rare mutation L68Q causes Hereditary Cystatin C Amyloid Angiopathy (HCCAA) [12,13], a condition in which the patients present fatal cerebral hemorrhage in early adulthood. *In vitro*, both WT and L68Q cystatins C produce amyloid fibrils [12,14,15]. Interestingly, *in vitro* and *in vivo* studies have shown that WT cystatin C binds to soluble A β protecting this peptide from oligomerization. In addition, cystatin C has been co-localized with A β deposits in senile plaques and the vascular cell wall. Altogether, these results suggest that cystatin C might be a neuroprotective protein in Alzheimer's disease (AD) acting as a modulator of amyloidosis [16–18].

In the eye, cystatin C is abundantly expressed by the retinal pigment epithelium (RPE) [19–21], the monolayer of cells essential for maintaining the

homeostasis of the neuroretina and the blood-retina barrier. RPE dysfunction is central to the pathogenesis of age-related macular degeneration (AMD), with various cellular processes contributing to the onset and progression of the disease including: cellular debris accumulation in drusen [22], changes to the underlying Bruch's membrane (BM) with and without breakage of the blood/retina barrier [23], impaired clearing of protein aggregates and/or damaged organelles [24,25].

The cystatin C variant type B has been associated as a recessive allele with increased risk of AD and exudative AMD [26–30]. This variant results from a SNP in the cystatin C gene (CST3) signal/leader sequence (1MAGPLRAPLLLLAILAVALAVSPAAG26) (p.Ala25Thr due to a c.G73A substitution). The 26-amino acid signal sequence of precursor cystatin C is essential for targeting the protein to the ER/Golgi apparatus and processing through the secretory pathway [31,32]. The A25T substitution decreases the efficiency of cleavage of the signal peptide in the variant B precursor resulting in a protein constituted of 146 amino acids that is abnormally processed in the cell [33]. The variant B precursor cystatin C is processed less efficiently through the secretory pathway resulting in its decreased secretion to the extracellular space, with some of the unprocessed molecules diverted from the secretory pathway accumulating intracellularly in association with the mitochondrial membrane network [33,34]. The reduced secretion and subsequent decreased concentration of mature cystatin C extracellularly could explain the enhanced AD and AMD risk observed in homozygous patients with variant B.

Here, due to its association to AD and AMD, we investigate the aggregation and amyloid propensity of the variant B, with emphasis on the signal peptide. Using two well validated aggregation predictors, namely AGGRESCAN, which was

designed to predict aggregation propensity regions *in vivo*, and Waltz, which predicts specific amyloid formation regions [35,36], we found on the immature form of the protein, four short aggregation-prone sequences, one of them being the leader peptide with very high aggregation/amyloidogenic scores. With these predictions in hands, we carried out *in vitro* experiments with the leader peptide to corroborate its amyloidogenicity. Our data unequivocally demonstrated that the leader peptide of the variant B cystatin C aggregates instantaneously upon aqueous dilution forming positive Thioflavin-T (Th-T) and Congo-red aggregates. ATR-FTIR, TEM and DLS confirmed the presence of amyloid in solution. The aggregates were shown to be very toxic to neuroblastoma cells cultures. In the light of these results, we propose for the first time a role of the leader peptide on the immature (unprocessed) form of variant B cystatin C in its association with increased risk of AD and AMD.

2. Materials and Methods

2.1. Prediction of the peptide aggregation and amyloid propensities

AGGRESCAN, Waltz, TANGO and Zipper DB Analysis - The intrinsic aggregation and amyloid formation propensities of mature and immature cystatin C were evaluated by these four methods employing default settings, using as input the primary sequence of either the mature protein or the immature form of variant B cystatin C. **Uniprot Accession Number P01034 (CYTC_HUMAN) and the dbSNP Classification code rs1064039 [Homo sapiens].**

2.2. Peptides synthesis

The peptides were purchased from CASLO ApS c/o Scion Denmark Technical Universit. The purity was higher than 98 %. The peptide was weighted and diluted in DMSO to obtain a stock solution of soluble peptide with a concentration of 3 mM.

2.3. Peptides Aggregation Assays

Aggregation was performed at different concentrations as stated in legends. Before each experiment, the stock solution was diluted in PBS pH 7.4 and Th-T was added at 20 μM (**Fig. 2B**) or 30 μM (**Fig. 2A**) final concentrations. The samples were incubated at 25°C with no agitation. Samples were excited at 450 nm while emission was collected from 400 to 600 nm on a spectrofluorometer Jasco 8200. Intensity at 482 nm was used to probe amyloid formation.

2.4. Congo red (CR) binding assay

The binding of CR to aggregated peptide was evaluated by diluting fivefold the incubated peptide in CR, resulting in a final CR concentration of 10 μM , and recording the absorbance spectra of the mixture in the 380 to 670 nm range in a Cary 400 spectrophotometer (Varian).

2.5. Dynamic Light Scattering (DLS) measurements

Peptide solutions were let to aggregate at the conditions stated above (100 μM) and measures were performed at 1 h and after 24 h of aggregation. DLS measurements were performed at 25 °C in a Malvern Zetasizer Nano S90 (Malvern, Worcestershire, UK). Each sample was measured three times; average distributions are presented.

2.6. Infrared spectroscopy

Attenuated total reflectance Fourier transform infrared spectroscopy (ATR FT-IR) was used to determine the secondary structure content of the aggregates formed at different times during the aggregation kinetics. The experiments were carried out using a Bruker Tensor 27 FT-IR spectrometer (Bruker Optics) with a Golden Gate MKII ATR accessory. Each spectrum consists of 16 accumulations measured at a resolution of 2 cm^{-1} in a wavelength range between 1,700 and 1,600 cm^{-1} . Infrared spectra were fitted through overlapping Gaussian curves, and the amplitude, mass center, bandwidth at half of the maximum amplitude, and area for each Gaussian function were calculated employing a nonlinear peak-fitting equation using the PeakFit package (Systat Software, San Jose, CA)

2.7. Transmission Electron Microscopy (TEM)

Aggregated samples were diluted in Mili-Q water to 10 μ M. 5 μ l of the solution were absorbed onto carbon-coated copper grids for 5 minutes and blotted to remove excess material. Uranyl acetate (2% w/v) was used for negative staining. Samples were dried on air for 5 min. Grids were exhaustively scanned with a Hitachi H-7000 transmission electron microscope operating at a voltage of 75 kV.

2.8. Neuroblastoma Cell (N2a) Culture and Viability Assay

N2a cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 2% antibiotic (gentamicin) in a 5% CO₂ atmosphere for 3 days and then transferred to a 96-well plate and allowed to adhere for 24 h (5,000 cells/well). Following cell adhesion, aggregates were added to achieve final concentration of 5 μ M. Cells were challenged for 24 h, and final cellular viability was measured by an MTT assay as previously described [37].

3. Results and Discussion

Bioinformatics predicts that the signal peptide of variant B cystatin C is aggregation-prone and amyloidogenic

AGGRESCAN is considered a reliable program to predict the aggregation propensity of proteins *in vivo* [38] since the aggregation score for each amino acid was derived from aggregation assays carried out on a bacterial system [35]. AGGRESCAN identifies aggregation-prone regions (APR) as stretches of a given sequence at least 5 amino acids long with high aggregation propensity scores [39].

Recently, the pentapeptide ⁴⁷LQVVR⁵¹ from cystatin C was demonstrated to form fibrils *in vitro* [40]. The same group by using the algorithm AMYLPRED identified two other peptides from the core of cystatin C with high aggregation propensity. These peptides, namely ⁵⁶IVAGVNYFLD⁶⁵ and ⁹⁵AFASFQIYAV¹⁰⁴ (it contains a C97A mutation), were also amyloidogenic upon incubation for several days at pH 5.5. These peptides numerations are from the mature cystatin C. In our **Figure 1B**, green numeration [41].

Figure 1C shows the aggregation propensity scores as predicted by AGGRESCAN for the immature (variant B) and mature forms of cystatin C. As seen, according to this algorithm, the mature protein contains three APRs located at positions 54-58 (DFAVG), 83-92 (VAGVNYFLDV) and 122-130 (FCSFQIYAV) (immature form numeration, orange numeration on **Figure 1B**). The regions 83-89 and 122-130 in the immature form correspond to residues 57-66 and 96-104 in the processed protein (green numeration on **Figure 1B**). Therefore, these two APRs almost completely overlap with those previously identified using AMYLPRED [42].

Interestingly, the signal peptide present in the immature (uncleaved) protein (segment 9-22; LLLAILAVALAVS) presented even higher aggregation scores than

the three previous peptides identified by AGGRESCAN. The primary sequence of precursor cystatin C, including the signal peptide, is presented in **Figure 1B** and the aggregation prone regions are highlighted.

APRs within the primary sequence of proteins are flanked by gatekeeper residues such as proline and charged amino acids, which modulate aggregation by opposing nucleation of aggregates [43,44]. In the case of the four stretches of cystatin C identified by AGGRESCAN, we could observe the presence of these gatekeeper residues. In particular, in the case of the signal peptide, the sequence is flanked by two proline residues at positions 8 and 23 (**P-9LLLLAILAVALAVS22-P**). Recently, our group identified the gatekeeper residues in the amyloidogenic protein transthyretin. By replacing one of these residues in the sequence of a peptide derived from TTR by a hydrophobic amino acid (K35L), we could convert a non-aggregating peptide into a highly aggregation-prone one [45].

Next, in order to investigate whether these APRs would also exhibit high scores for forming amyloid-specific aggregates, the Waltz program was utilized (**Fig. 1D**). As seen, all four segments of cystatin C, including the signal peptide (segment 10-20 as predicted by this algorithm), presented propensity to form amyloid assemblies. In this case, the other identified regions with this property were 56-61 (AVGEYN), 84-92 (AGVNYFLDV) and 124-130 (SFQIIYAV) (immature form numeration, orange numeration on **Figure 1B**). Both analyses were performed with the two protein sequences, but as the only difference present between them is the signal peptide, solid and dashed lines appear superimposed.

We also used other algorithms such as TANGO [46], Zipper DB [47] and AMYLPRED2 to analyze the aggregation and amyloid propensity of the cystatin C signal peptide. TANGO and AMYLPRED2 predicted the 9-20 and 9-21 stretches to

be aggregation-prone, respectively, whereas Zipper DB predicted the sequence 9-17 to be highly amyloidogenic. Because the methods we employed here for computational predictions rely on very different principles, the prediction of the signal peptide being highly aggregation prone and amyloidogenic was considered to be very robust. The intrinsic high hydrophobicity of signal peptides does not always implies that they can form typical beta sheet rich ordered amyloid fibrils, as cystatin C signal peptide does. To strengthen this very important point, we ran Waltz for 20 signal peptides of different human proteins randomly chosen from Uniprot (data not shown). Interestingly, only 2 out of 20 presented regions that could drive amyloid formation (Waltz negative: P30453, P30483, Q9TNN7, P04114, P25774, P08185, P16870, P11597, P28325, Q9UBU2, Q9NR61, P16444, P54803, P47871, Q6UWU2, P42262; Waltz Positive: Q8NFBZ8, P12111).

Variant B Cystatin C signal peptide is able to form amyloid fibrils *in vitro*

Having *in silico* evidence that the signal peptide presents high aggregation propensity and amyloidogenicity, we synthesized the peptide with the mutation A25T (1MAGPLRAPLLLLAILAVALAVSPATG26) (**Fig. 1B**) and incubated it at increasing concentrations (5 μ M to 100 μ M) for 1 h at pH 7.4, 25°C under stagnant conditions. As shown in **Figure 2A**, there was a proportional increase in Thioflavin-T (Th-T) emission at increasing concentrations of the peptide, suggesting formation of amyloid-like aggregates in solution. Congo red (CR) binding assays were also performed to confirm the presence of amyloid-like aggregates (**Fig. 2A, inset**).

In order to follow the aggregation kinetics of the signal peptide, increasing concentrations of the peptide were incubated with Th-T and its fluorescence emission was collected over time (**Fig. 2B**). Notably, even at very low peptide concentration

such as 5 μ M, maximum emission of Th-T was attained in the first acquisition points, with no further change in the fluorescence signal of the probe even after 3 days under aggregation conditions. This suggests that aggregation of the signal peptide of cystatin C is very fast. We tried several conditions to slow down the aggregation process of the signal peptide such as low temperature (4 and 8°C) and addition of 5, 10 and 15% DMSO to the aggregating buffer without any success (not shown).

In order to confirm the instantaneous aggregation of the signal peptide, a suspension with 50 μ M peptide diluted into 30 μ M Th-T was filtered through a 0.22 micrometer filter before measurements. The filter became yellow due to the retention of the aggregates bound to Th-T. The filtered solution, when let to aggregate, was not able to bind additional Th-T since all soluble peptide was converted into amyloid aggregates that were retained in the filter (**Fig. 2B, dashed line**).

Characterizing the morphology, secondary structure and toxicity of the aggregates formed by the signal peptide of variant B cystatin C

Next, we used TEM to study the morphology of the species present at different times of aggregation (**Fig. 3A**). Interestingly, although Th-T binding has already leveled-off at 1 h under aggregation conditions, the great majority of the aggregated material presented an amorphous appearance and only few amyloid fibrils were observed at this time (**Fig. 3A, left**). The binding of Th-T to these amorphous species suggests that they already present cross- β fold able to accommodate this probe. These species are on-pathway to fibril formation since after 72 h under aggregation condition only mature fibrils were observed by TEM (**Fig. 3A, right**). At 24 h, it was already possible to see the presence of fibrils but small amorphous aggregates still remained (**Fig. 3A, middle**).

In order to get insights into the secondary structural changes that take place with the signal peptide upon aggregation, we took advantage of ATR-FTIR (**Fig. 3 B**). Left panel shows the FTIR spectra of the peptide dried from a stock solution with 100% DMSO (continuous line) and those collected during the first hour of aggregation. As seen, in DMSO the peptide presented a unique and broad peak centered at $1,660\text{ cm}^{-1}$, which corresponds to disordered, random coiled structures, which is a strong evidence that the peptide is monomeric in DMSO [48–50].

In the initial times of aggregation (up to 1 h), the spectra changed and a new peak at $1,628\text{ cm}^{-1}$ appeared, which was assigned to β -sheet structures. At the same time, the peak related to random-coil structures ($1,660\text{ cm}^{-1}$) decreased but was still present. The presence of β -sheet structures in these initial aggregates explains why these species bind Th-T (as shown in **Fig. 2**), but they are not fully organized, as seen by TEM (**Fig. 3A, left**).

As aggregation proceeded for longer times (16, 24 and 72 h), the peak of β -sheet ($1,627\text{ cm}^{-1}$) increased even further, with a concomitant decrease of the peak of random coil at $1,660\text{ cm}^{-1}$. **Table 1** summarizes the secondary structural changes that take place upon aggregation of the signal peptide of cystatin C.

Next, to further confirm the difference in sizes of the species formed at 1 and 24 h, DLS measurements were performed (**Fig. 3C**). The main sizes of the two more prevalent species present at 1 h were $0.194\text{ nm} \pm 0.06\text{ nm}$ and $904 \pm 32.14\text{ nm}$. After 24 h under aggregation conditions, there were two species present, one with $947 \pm 134\text{ nm}$ and an extreme large species with $3,008 \pm 500\text{ nm}$. Interestingly and corroborating the FTIR data, the species with around 900 nm were still present at 24 h. Given the fact that the aggregation pathways of aggregation-prone proteins are distinct leading to the accumulation of a myriad of aggregated species with different size and

morphologies, it is difficult to make a direct comparison of the species sizes observed here with others. However, initial fibrillar species from A β peptide have been shown to have sizes from 200 to 700 nm [51,52]. In another study [53], the formation of transthyretin fibrillar oligomers of 1,000 nm and mature fibrils with mean size of 4,000 nm was reported [54], in accordance with our DLS data where early aggregating species with 900 nm and mature fibrils with 3,000 nm were detected. We performed a calibration assay using a standard solution of silicone particles and the expected size was found (342 nm).

Next, neuroblastoma cell line N2a was used to probe the cytotoxicity of the aggregates formed at 1 and 24 h and cell viability was evaluated by MTT assays. Both aggregates tested at 5 μ M concentration were very cytotoxic, killing ~40 % of the cells (**Fig. 3D**).

Conclusions

The substitution A25T in the signal peptide of the variant B precursor cystatin C compromises its cleavage and/or processing precluding the efficient formation of the mature form of the protein, which is secreted from the cells to the extracellular milieu where it exerts its biochemical effect as a cysteine protease inhibitor. We have modeled the potential impact of the Ala to Thr mutation with AGGRESCAN, Tango and Waltz. Neither Tango nor Waltz detected any change in the extension of the aggregation-prone regions or in the overall predicted aggregation/amyloid propensity. AGGRESCAN did not identify any change in the extension of the aggregation-prone regions and the increase in overall aggregation propensity was only of 0.7%. A model of the functional consequences for trafficking and protein aggregation of this variant precursor cystatin C is presented in **Figure 4**. As previously shown, in the

extracellular space mature cystatin C interacts with A β diminishing its aggregation [17,18]. An inefficiently cleaved/processed variant B cystatin C leads on one hand to a significantly reduced secretion of the mature cystatin C as shown previously [33,34] and, on the other, to the accumulation of full length variant precursor protein containing the mutated, highly amyloidogenic leader. On the contrary, the WT signal peptide is excised from the normal cystatin c protein during its processing and should be completely cleaved. To our knowledge, there is no description of the accumulation/aggregation of the WT signal peptide inside RPE cells. In the light of our data, it is tempting to suggest that this variant might aggregate into amyloid fibrils through the leader peptide, since the other stretches with high aggregation propensity are protected in the globular domain of the protein. These aggregates composed of uncleaved/unprocessed variant B could be formed either inside or outside the cells leading to a gain of toxic function. As demonstrated by Paraoan *et al.*, 2004, this variant is retained close to mitochondria walls forming structures resistant to protease digestion [34], a characteristic feature of high molecular weight aggregates, including amyloid fibrils. Upon aggregation of variant B, other important intra- or extracellular protein components could be incorporated into this aggregated material, possibly depriving the cell/tissue from a specific function and forming protein aggregates otherwise not present in the cells. Additionally, low levels of functional mature cystatin C in the extracellular space could be very deleterious, contributing to increased proteolytic action at its extracellular sites of activity. Also, there will be less available protein to interact with A β peptide, thus enhancing its aggregation, which may explain the variant B cystatin C association with a higher risk of developing AD.

As the amyloid deposits are the histological hallmark of AD, drusen are the extracellular deposits found in AMD. The presence of A β peptide has been reported

in patients with severe forms of AMD [55–58]. The involvement of protein aggregation in AMD pathogenesis is suggested by the presence of various toxic amyloid structures in drusen [56,59] and, indirectly, by the decrease of the AMD risk obtained through anti-amyloid treatment in a mouse model [60]. Variant B cystatin C aggregation may therefore contribute to AMD pathogenesis. In addition, if this mutation avoids the observed partnership between cystatin C and A β peptide it can account for the higher incidence of AD in homozygous patients. Further work is being carried out to investigate amyloid deposits formed by this variant inside RPE cells. More experimental approaches are necessary to reveal in full the mechanism of action of variant B cystatin C in these major neurodegenerative diseases and we are actively pursuing such studies. With the actual lack of any *in vitro* aggregation experimental evidence to link the higher incidence of AD and AMD with variant B, we believe that the present study provides the important first clues for further investigations.

Authors Contribution

DF and LP conceived and supervised the study; RS, SV, DF and LP designed experiments; RS and SN performed experiments; LP and SV provided tools and reagents; RS, DF, LP, SN and SV analyzed data; RS, DF and LP wrote the manuscript; SV, LP and DF made manuscript revisions.

Conflict of interest statement

The authors declare no conflict of interest.

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FIGURE LEGENDS

Fig. 1. Tridimensional structure, primary sequence and in silico aggregation and amyloid propensity predictions for WT and variant B cystatin C. (A) A monomer-stabilized human cystatin C with an engineered disulfide bond (PDB ID: 3GAX) [61] reveals the canonical cystatin fold, based on the crystal structure of chicken cystatin [62]. In red are the regions pointed out by AGGRESCAN as the aggregation prone regions in the protein. (B) The amino acid sequence of the variant B precursor cystatin C with the aggregation-prone regions (APR) identified by AGGRESCAN in **bold** and by Waltz underlined. The leader peptide is highlighted in yellow. Gatekeeper residues flanking APR of the signal peptide are in blue. We are using two numerations, the orange numbering includes the signal peptide and contains 146 residues, the green numeration corresponds to the processed cystatin C, which contains 120 residues. In (C), the aggregation propensity of both sequences was predicted using AGGRESCAN (<http://bioinf.uab.es/aggrescan/>) using default parameters. (D) The same procedure was used to predict the amyloid propensity by the algorithm Waltz (<http://www.switchlab.org/bioinformatics/waltz>). We call attention to the fact that continuous lines (mature cystatin C) are superimposed to the dashed lines (immature cystatin C).

Fig. 2. Monitoring aggregation of variant B cystatin C signal peptide *in vitro*.

Panel A: Increasing concentrations of the signal peptide (5 to 100 μ M, as indicated) were incubated in PBS (pH 7.4) at 25°C without agitation (aggregation condition). After 1 h under this condition, Th-T binding was evaluated by measuring its fluorescence emission (Excitation = 450 nm and Emission = 450 to 600 nm). [Th-T] =

20 μ M. The inset in Panel A shows the spectra of CR alone and in the presence of aggregates formed after 24 h of incubation. **Panel B** displays the kinetics of aggregation at different concentrations of the signal peptide (see the legend inside this panel) as followed by the intensity of Th-T fluorescence at 482. [Th-T] = 30 μ M

Fig. 3. Characterization of morphology, secondary structure content, size distribution and toxicity of the aggregates formed by the variant B cystatin C signal peptide. (A) Signal peptide at 100 μ M was left to aggregate under the conditions described in Fig. 2. At 1 h (left images), 24 h (middle images) and 72 h (right images) aliquots were withdrawn and imaged by TEM. Bars represent 100, 200 and 500 nm. (B) Secondary structure changes that take place upon aggregation of the signal peptide analyzed by ATR-FTIR. The spectra displayed on the left side are those of the soluble peptide (full line), and of the samples aggregated up to 1 h as follows: 5 min (dotted), 15 min (short dash), 40 min (dash) and 60 min (large dash). On the right are displayed the FTIR spectra of the samples aggregated for longer times as follows: 1 h (full), 16 h (dot), 24 h (short dash) and 72 h (dash-dot). Secondary structure quantification from the deconvolution of these spectra is displayed in **Table 1**. (C) DLS was used to determine the size of the main species present at 1 h (dash-dot line) and 24 h (full line) under aggregating conditions. A calibration with silicone particles was performed and is shown in dotted line. (D) Toxicity of the aggregates formed at 1 and 24 h on neuroblastoma cells determined by MTT assay. In each case, 5 μ M of aggregated material was added to the cells in culture remaining in contact with them for 24 h before cell viability evaluation.

Fig. 4. Schematic representation of the effects of the A25T mutation on variant B precursor cystatin C cell trafficking and its possible implications for protein aggregation. On the left, a RPE cell that produces WT cystatin C. Processing of cystatin C takes place normally in the ER/Golgi network. The signal peptide is cleaved leading to the formation of the mature form of the protein, which is secreted from the RPE cells. Outside the cell, cystatin C acts as inhibitor of cysteine proteases. Besides, its can interact with A β modulating its aggregation properties. On the right, a

RPE cell that produces the variant B, which is abnormally processed accumulating in the periphery of the mitochondria, probably as an aggregated material induced by the presence of the signal peptide. This variant is inefficiently secreted what impairs its protective effect against A β aggregation.

Bibliography

- [1]Grubb A & Löfberg H (1982) Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. *Proc. Natl. Acad. Sci. U. S. A.* **79**, 3024–7.
- [2]Abrahamson M, Alvarez-Fernandez M & Nathanson C-M (2003) Cystatins. *Biochem. Soc. Symp.*, 179–99.
- [3]Turk V & Bode W (1991) The cystatins: protein inhibitors of cysteine proteinases. *FEBS Lett.* **285**, 213–9.
- [4]Engh RA, Dieckmann T, Bode W, Auerswald EA, Turk V, Huber R & Oschkinat H (1993) Conformational Variability of Chicken Cystatin. *J. Mol. Biol.* **234**, 1060–1069.
- [5]Tavéra C, Leung-Tack J, Prévot D, Gensac MC, Martinez J, Fulcrand P & Collé A (1992) Cystatin C secretion by rat glomerular mesangial cells: autocrine loop for in vitro growth-promoting activity. *Biochem. Biophys. Res. Commun.* **182**, 1082–8.
- [6]Hasegawa A, Naruse M, Hitoshi S, Iwasaki Y, Takebayashi H & Ikenaka K (2007) Regulation of glial development by cystatin C. *J. Neurochem.* **100**, 12–22.
- [7]Bobek LA & Levine MJ (1992) Cystatins--inhibitors of cysteine proteinases. *Crit. Rev. Oral Biol. Med.* **3**, 307–32.
- [8]Warfel AH, Zucker-Franklin D, Frangione B & Ghiso J (1987) Constitutive secretion of cystatin C (gamma-trace) by monocytes and macrophages and its downregulation after stimulation. *J. Exp. Med.* **166**, 1912–7.
- [9]Lerner UH & Grubb A (2009) Human cystatin C, a cysteine proteinase inhibitor, inhibits bone resorption in vitro stimulated by parathyroid hormone and parathyroid hormone-related peptide of malignancy. *J. Bone Miner. Res.* **7**, 433–440.
- [10]Yamada M (2000) Cerebral amyloid angiopathy: an overview. *Neuropathology* **20**, 8–22.
- [11]Grubb AO (2000) Cystatin C--properties and use as diagnostic marker. *Adv. Clin. Chem.* **35**, 63–99.
- [12]Palsdottir A, Snorraddottir AO & Thorsteinsson L (2006) Hereditary cystatin C amyloid angiopathy: genetic, clinical, and pathological aspects. *Brain Pathol.* **16**, 55–9.
- [13]Palsdottir A, Helgason A, Palsson S, Bjornsson HT, Bragason BT, Gretarsdottir S, Thorsteinsdottir U, Olafsson E & Stefansson K (2008) A drastic reduction in the life span of cystatin C L68Q carriers due to life-style changes during the last

- two centuries. *PLoS Genet.* **4**, e1000099.
- [14]Nagai A, Kobayashi S, Shimode K, Imaoka K, Umegae N, Fujihara S & Nakamura M (1998) No mutations in cystatin C gene in cerebral amyloid angiopathy with cystatin C deposition. *Mol. Chem. Neuropathol.* **33**, 63–78.
- [15]van Duinen SG, Castaño EM, Prelli F, Bots GT, Luyendijk W & Frangione B (1987) Hereditary cerebral hemorrhage with amyloidosis in patients of Dutch origin is related to Alzheimer disease. *Proc. Natl. Acad. Sci. U. S. A.* **84**, 5991–4.
- [16]Sastre M, Calero M, Pawlik M, Mathews PM, Kumar A, Danilov V, Schmidt SD, Nixon RA, Frangione B & Levy E (2004) Binding of cystatin C to Alzheimer's amyloid beta inhibits in vitro amyloid fibril formation. *Neurobiol. Aging* **25**, 1033–43.
- [17]Mi W, Pawlik M, Sastre M, Jung SS, Radvinsky DS, Klein AM, Sommer J, Schmidt SD, Nixon RA, Mathews PM & Levy E (2007) Cystatin C inhibits amyloid-beta deposition in Alzheimer's disease mouse models. *Nat. Genet.* **39**, 1440–2.
- [18]Gauthier S, Kaur G, Mi W, Tizon B & Levy E (2011) Protective mechanisms by cystatin C in neurodegenerative diseases. *Front. Biosci. (Schol. Ed.)*. **3**, 541–54.
- [19]Paraoan L, Grierson I & Maden BE (2000) Analysis of expressed sequence tags of retinal pigment epithelium: cystatin C is an abundant transcript. *Int. J. Biochem. Cell Biol.* **32**, 417–26.
- [20]Ida H, Boylan SA, Weigel AL, Smit-McBride Z, Chao A, Gao J, Buchoff P, Wistow G & Hjelmeland LM (2004) EST analysis of mouse retina and RPE/choroid cDNA libraries. *Mol. Vis.* **10**, 439–44.
- [21]Wistow G, Bernstein SL, Wyatt MK, Fariss RN, Behal A, Touchman JW, Bouffard G, Smith D & Peterson K (2002) Expressed sequence tag analysis of human RPE/choroid for the NEIBank Project: over 6000 non-redundant transcripts, novel genes and splice variants. *Mol. Vis.* **8**, 205–20.
- [22]Anderson DH, Mullins RF, Hageman GS & Johnson L V (2002) A role for local inflammation in the formation of drusen in the aging eye. *Am. J. Ophthalmol.* **134**, 411–31.
- [23]Booij JC, Baas DC, Beisekeeva J, Gorgels TGMF & Bergen AAB (2010) The dynamic nature of Bruch's membrane. *Prog. Retin. Eye Res.* **29**, 1–18.
- [24]Mitter SK, Rao HV, Qi X, Cai J, Sugrue A, Dunn WA, Grant MB & Boulton ME (2012) Autophagy in the retina: a potential role in age-related macular degeneration. *Adv. Exp. Med. Biol.* **723**, 83–90.
- [25]Kaarniranta K, Sinha D, Blasiak J, Kauppinen A, Veréb Z, Salminen A, Boulton ME & Petrovski G (2013) Autophagy and heterophagy dysregulation leads to

- retinal pigment epithelium dysfunction and development of age-related macular degeneration. *Autophagy* **9**, 973–84.
- [26]Hua Y, Zhao H, Lu X, Kong Y & Jin H (2012) Meta-Analysis of the Cystatin C(CST3) Gene G73A Polymorphism and Susceptibility to Alzheimer’s Disease. *Int. J. Neurosci.*
- [27]Butler JM, Sharif U, Ali M, McKibbin M, Thompson JP, Gale R, Yang YC, Inglehearn C & Paraoan L (2015) A missense variant in CST3 exerts a recessive effect on susceptibility to age-related macular degeneration resembling its association with Alzheimer’s disease. *Hum. Genet.* **134**, 705–15.
- [28]Paraoan L, Hiscott P, Gosden C & Grierson I (2010) Cystatin C in macular and neuronal degenerations: Implications for mechanism(s) of age-related macular degeneration. *Vision Res.* **50**, 737–742.
- [29]Finckh U, von der Kammer H, Velden J, Michel T, Andresen B, Deng A, Zhang J, Müller-Thomsen T, Zuchowski K, Menzer G, Mann U, Papassotiropoulos A, Heun R, Zurdel J, Holst F, Benussi L, Stoppe G, Reiss J, Miserez AR, Staehelin HB, Rebeck GW, Hyman BT, Binetti G, Hock C, Growdon JH & Nitsch RM (2000) Genetic association of a cystatin C gene polymorphism with late-onset Alzheimer disease. *Arch. Neurol.* **57**, 1579–83.
- [30]Zurdel J, Finckh U, Menzer G, Nitsch RM & Richard G (2002) CST3 genotype associated with exudative age related macular degeneration. *Br. J. Ophthalmol.* **86**, 214–9.
- [31]Paraoan L, White MR, Spiller DG, Grierson I & Maden BE Precursor cystatin C in cultured retinal pigment epithelium cells: evidence for processing through the secretory pathway. *Mol. Membr. Biol.* **18**, 229–36.
- [32]Paraoan L, Grierson I & Maden BEH (2003) Fate of cystatin C lacking the leader sequence in RPE cells. *Exp. Eye Res.* **76**, 753–6.
- [33]Benussi L, Ghidoni R, Steinhoff T, Alberici A, Villa A, Mazzoli F, Nicosia F, Barbiero L, Broglio L, Feudatari E, Signorini S, Finckh U, Nitsch RM & Binetti G (2003) Alzheimer disease-associated cystatin C variant undergoes impaired secretion. *Neurobiol. Dis.* **13**, 15–21.
- [34]Paraoan L, Ratnayaka A, Spiller DG, Hiscott P, White MRH & Grierson I (2004) Unexpected intracellular localization of the AMD-associated cystatin C variant. *Traffic* **5**, 884–95.
- [35]Conchillo-Solé O, de Groot NS, Avilés FX, Vendrell J, Daura X & Ventura S (2007) AGGRESKAN: a server for the prediction and evaluation of “hot spots” of aggregation in polypeptides. *BMC Bioinformatics* **8**, 65.
- [36]Oliveberg M (2010) Waltz, an exciting new move in amyloid prediction. *Nat.*

Methods **7**, 187–8.

- [37]Malmo C, Vilasi S, Iannuzzi C, Tacchi S, Cametti C, Irace G & Sirangelo I (2006) Tetracycline inhibits W7FW14F apomyoglobin fibril extension and keeps the amyloid protein in a pre-fibrillar, highly cytotoxic state. *FASEB J.* **20**, 346–7.
- [38]Belli M, Ramazzotti M & Chiti F (2011) Prediction of amyloid aggregation in vivo. *EMBO Rep.* **12**, 657–63.
- [39]Sánchez de Groot N, Pallarés I, Avilés FX, Vendrell J & Ventura S (2005) Prediction of “hot spots” of aggregation in disease-linked polypeptides. *BMC Struct. Biol.* **5**, 18.
- [40]Tsiolaki PL, Hamodrakas SJ & Iconomidou VA (2015) The pentapeptide LQVVR plays a pivotal role in human cystatin C fibrillization. *FEBS Lett.* **589**, 159–64.
- [41]Tsiolaki PL, Louros NN, Hamodrakas SJ & Iconomidou VA (2015) Exploring the “aggregation-prone” core of human Cystatin C: A structural study. *J. Struct. Biol.* **191**, 272–280.
- [42]Tsolis AC, Papandreou NC, Iconomidou VA & Hamodrakas SJ (2013) A consensus method for the prediction of “aggregation-prone” peptides in globular proteins. *PLoS One* **8**, e54175.
- [43]Reumers J, Maurer-Stroh S, Schymkowitz J & Rousseau F (2009) Protein sequences encode safeguards against aggregation. *Hum. Mutat.* **30**, 431–7.
- [44]Richardson JS & Richardson DC (2002) Natural beta-sheet proteins use negative design to avoid edge-to-edge aggregation. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 2754–9.
- [45]Sant’Anna R, Braga C, Varejão N, Pimenta KM, Graña-Montes R, Alves A, Cortines J, Cordeiro Y, Ventura S & Foguel D (2014) The importance of a gatekeeper residue on the aggregation of transthyretin: implications for transthyretin-related amyloidoses. *J. Biol. Chem.* **289**, 28324–37.
- [46]Fernandez-Escamilla A-M, Rousseau F, Schymkowitz J & Serrano L (2004) Prediction of sequence-dependent and mutational effects on the aggregation of peptides and proteins. *Nat. Biotechnol.* **22**, 1302–6.
- [47]Iwata K, Fujiwara T, Matsuki Y, Akutsu H, Takahashi S, Naiki H & Goto Y (2006) 3D structure of amyloid protofilaments of beta2-microglobulin fragment probed by solid-state NMR. *Proc. Natl. Acad. Sci. U. S. A.* **103**, 18119–24.
- [48]Byler DM & Susi H (1986) Examination of the secondary structure of proteins by deconvolved FTIR spectra. *Biopolymers* **25**, 469–87.
- [49]Barth A & Zscherp C (2002) What vibrations tell us about proteins. *Q. Rev.*

Biophys. **35**, 369–430.

- [50]Jackson M & Mantsch HH (1991) Protein secondary structure from FT-IR spectroscopy: correlation with dihedral angles from three-dimensional Ramachandran plots. *Can. J. Chem.* **69**, 1639–1642.
- [51]Merz PA, Wisniewski HM, Somerville RA, Bobin SA, Masters CL & Iqbal K (1983) Ultrastructural morphology of amyloid fibrils from neuritic and amyloid plaques. *Acta Neuropathol.* **60**, 113–24.
- [52]Bitan G, Kirkitadze MD, Lomakin A, Vollers SS, Benedek GB & Teplow DB (2003) Amyloid beta -protein (Abeta) assembly: Abeta 40 and Abeta 42 oligomerize through distinct pathways. *Proc. Natl. Acad. Sci. U. S. A.* **100**, 330–5.
- [53]Ferreira N, Saraiva MJ & Almeida MR (2011) Natural polyphenols inhibit different steps of the process of transthyretin (TTR) amyloid fibril formation. *FEBS Lett.* **585**, 2424–30.
- [54]Ferreira N, Saraiva MJ & Almeida MR (2012) Natural polyphenols as modulators of TTR amyloidogenesis: in vitro and in vivo evidences towards therapy. *Amyloid* **19 Suppl 1**, 39–42.
- [55]Dentchev T, Milam AH, Lee VM-Y, Trojanowski JQ & Dunaief JL (2003) Amyloid-beta is found in drusen from some age-related macular degeneration retinas, but not in drusen from normal retinas. *Mol. Vis.* **9**, 184–90.
- [56]Luibl V, Isas JM, Kaye R, Glabe CG, Langen R & Chen J (2006) Drusen deposits associated with aging and age-related macular degeneration contain nonfibrillar amyloid oligomers. *J. Clin. Invest.* **116**, 378–85.
- [57]Anderson DH, Talaga KC, Rivest AJ, Barron E, Hageman GS & Johnson L V (2004) Characterization of beta amyloid assemblies in drusen: the deposits associated with aging and age-related macular degeneration. *Exp. Eye Res.* **78**, 243–56.
- [58]Johnson L V, Leitner WP, Rivest AJ, Staples MK, Radeke MJ & Anderson DH (2002) The Alzheimer's A beta -peptide is deposited at sites of complement activation in pathologic deposits associated with aging and age-related macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* **99**, 11830–5.
- [59]Isas JM, Luibl V, Johnson L V, Kaye R, Wetzel R, Glabe CG, Langen R & Chen J (2010) Soluble and mature amyloid fibrils in drusen deposits. *Invest. Ophthalmol. Vis. Sci.* **51**, 1304–10.
- [60]Ding J-D, Johnson L V, Herrmann R, Farsiu S, Smith SG, Groelle M, Mace BE, Sullivan P, Jamison JA, Kelly U, Harrabi O, Bollini SS, Dilley J, Kobayashi D, Kuang B, Li W, Pons J, Lin JC & Bowes Rickman C (2011) Anti-amyloid

therapy protects against retinal pigmented epithelium damage and vision loss in a model of age-related macular degeneration. *Proc. Natl. Acad. Sci. U. S. A.* **108**, E279–87.

- [61] Kolodziejczyk R, Michalska K, Hernandez-Santoyo A, Wahlbom M, Grubb A & Jaskolski M (2010) Crystal structure of human cystatin C stabilized against amyloid formation. *FEBS J.* **277**, 1726–37.
- [62] Bode W, Engh R, Musil D, Thiele U, Huber R, Karshikov A, Brzin J, Kos J & Turk V (1988) The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. *EMBO J.* **7**, 2593–9.

Table 1. Secondary structure changes that take place upon aggregation of variant B cystatin C signal peptide measured by FTIR.

	Inter β-sheet (%)	Disordered (%)	β-sheet (%)
Time	1623 – 1641 cm^{-1}	1642 – 1657 cm^{-1}	1674 – 1695 cm^{-1}
0	-	100	-
5 min	42.98	37.44	19.57
15 min	42.07	39.56	18.36
40 min	41.64	37.23	21.11
1 h	41.78	38.42	19.79
16 h	64.02	35.98	-
24 h	83.55	16.43	-
72 h	86.22	13.76	-

